Recommended Protocols for Microbiological Studies in Puget Sound

For

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INTRODUCTION

This chapter presents recommendations for measuring microorganisms in Puget Sound. The recommendations are based on the results of a workshop and on written reviews by representatives from most organizations that fund or conduct microbiological studies in the Sound (Table 1). The purpose of developing these recommendations is to encourage all Puget Sound investigators conducting monitoring programs, baseline surveys, and intensive investigations to use standardized methods whenever possible. If this goal is achieved, most data collected in Puget Sound should be directly comparable and thereby capable of being integrated into a sound-wide database. Such a database is necessary for developing and maintaining a comprehensive water quality management program for Puget Sound.

The initial section of this chapter describes those microorganisms currently being measured in Puget Sound. In subsequent sections recommended microorganisms for future studies are identified, and special considerations for sampling water, sediment, and tissue are discussed. Finally, the uses and limitations of the recommended microorganisms are discussed, and laboratory and quality assurance/quality control (QA/QC) procedures are recommended.

It is recognized that departures from the general recommendations made herein may be necessary to meet the special requirements of individual projects. If such departures are made, however, the funding agency or investigator should be aware that the resulting data may not be comparable with most other data of that kind. In some instances, data collected using different methods may be compared if the methods are intercalibrated adequately.

TABLE 1. CONTRIBUTORS TO THE MICROBIOLOGY

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MICROBIOLOGICAL MEASUREMENTS CURRENTLY MADE IN PUGET SOUND

A variety of agencies sample for bacteria in the water, sediment, or biota of Puget Sound. These agencies and the various bacterial indicators (i.e., of pathogens) and/or primary pathogens they evaluate are listed in Table 2. Measurements of bacterial indicators that are currently used include:

- Total counts of coliform bacteria (i.e., total coliform bacteria)
- Counts of fecal coliform bacteria (i.e., fecal coliform bacteria)
- Counts of Escherichia coli as a fraction of fecal coliform bacteria (i.e., fecal coliform bacteria/E. coli)
- Counts of enterococci, a subset of fecal streptococci (i.e., enterococci)
- Counts of Clostridium perfringens (i.e., C. perfringens)
- Counts of heterotrophic bacteria by standard plate count methods (i.e., standard plate counts).

TABLE 2. CURRENT BACTERIOLOGICAL MEASUREMENTS IN PUGET SOUND[a]

	Routine Monitoring[b]		Special Projects
Agency	**Water**	**Tissue**	
U.S. FDA (Seattle)	Total coliform bacteria (I) Fecal coliform bacteria (I) Fecal streptococci (I,NR)	Total coliform bacteria (I) Fecal coliform bacteria (I)	Vibrio dpp. (P) C. perfringens (P) Aeromonas hydrophilia (I,P) Versinis spp. (P)
U.S. EPA (Region 10)	—	—	Occasional (most work is in fresh water)[c]
Seattle-King Co. Health Dept.	—	Fecal coliform bacteria (I)(NR)	
Wash. Dept. of Ecology	Total coliform bacteria (I) Fecal coliform bacteria (I)	—	
METRO	Fecal coliform bacteria (I)	Fecal coliform bacteria (I)	C. perfringens (I,P) E. coli (I) Vibrio parahaemolyticus (P) Enterococci (I)
Wash. Dept. of Social and Health Services	Fecal coliform bacteria (I)	Fecal coliform bacteria (I) Standard plate counts (I)	

a I = indicator of primary pathogens or specific pollution sources, P = primary pathogen, NR = not routine.

b No agency in Puget Sound conducts routine monitoring of bacteria in sediments.

c Marine studies have included the fate and survival of fecal coliform bacteria and heterotrophic bacteria in relation to dredging operations.

RECOMMENDATIONS FOR FUTURE STUDIES

BACTERIAL INDICATORS

Reliance on a single bacterial indicator or a suite of indicators may not be desirable as a general rule because the objectives of different studies may vary substantially. For example, requirements for water pollution monitoring differ from those for evaluating the acceptability of shellfish for human consumption. In general, however, the following bacterial indicators should be considered for use in most surveys of bacterial indicators in Puget Sound:

- Fecal coliform bacteria
- Fecal coliform bacteria/E. coli
- Enterococci
- C. perfringens.

These indicators might be used individually or in various combinations depending upon the specific objectives of each study. The best uses and the limitations of each indicator are discussed later in this chapter.

PRIMARY PATHOGENS

In some cases, disease outbreaks may require the direct investigation and identification of primary pathogens because use of an indicator is inappropriate. For example, outbreaks of acute gastroenteritis may show no correspondence between the presence of a specific indicator, such as fecal coliform bacteria and the primary pathogen, such as Yersinia enterocolitica (Munger et al. 1980). Direct identification of primary pathogens may also be appropriate during reconnaissance surveys in areas lacking historical data. The choice of pathogen should be determined by the nature of the disease and by seasonal considerations. For example, Vibrio bacteria generally are found in the warmer months, whereas Yersinia bacteria are more prevalent during colder periods of the year.

SPECIAL SAMPLING CONSIDERATIONS

Choice of an optimum matrix for sampling (e.g., water column, sediment, interstitial water, or tissue) will depend upon the objectives of each study. For example, public health impacts are most likely through ingestion of contaminated shellfish or contact with contaminated water. Shellfish tissues or recreational waters may therefore be the materials most appropriate for analysis in studies focusing on health risks. The identification of present point sources of pollution can best be determined by analysis of water samples, whereas long-term pollution trends may best be described by sediment analysis.

WATER COLUMN

Water sampling can result in highly variable data because bacteria are not uniformly distributed throughout the water column (Gameson 1983) and sample volumes generally are limited to 50-100 mL. One major cause of spatial heterogeneity is the tendency for bacterial cells to concentrate in a thin microlayer on the surface of the water. Because bacterial abundances in the microlayer may exceed abundances in underlying surface water by several orders of magnitude (Hardy 1982), it is recommended that the microlayer and underlying water be sampled separately. However, sampling of the microlayer requires specialized techniques that have yet to be standardized. Also, collection and analysis of samples from both the microlayer and underlying water at each station may be too expensive for many routine monitoring programs. Thus, if separate samples cannot be collected within the constraints of a particular program, it is recommended that the microlayer be included in the sample by using the traditional "scoop" method of surface water sampling (U.S. EPA 1978). This method involves plunging an open bottle straight down to a depth of 15-30 cm below the water surface, moving it horizontal to the surface while tipping it slightly to let trapped air escape, and removing the bottle in a vertical position. It is recommended that samples be collected using a wide-mouth (12-15 cm) bottle to facilitate inclusion of the microlayer.

SEDIMENTS

Sediments are known to be heterogeneous with respect to types and numbers of bacteria. In addition, the bacteriological composition of sediments may have little relationship to public health impacts. For these reasons, routine monitoring of sediments in Puget Sound presently is not undertaken by any organization (Table 1), and is not recommended except under special circumstances.

One special application of sediment monitoring is analysis for C. perfringens to trace the distribution of sewage. Because spores of this bacterium are associated with fecal pollution and survive over long periods, they offer the advantage of providing a cumulative record of sewage influence suitable for long-term monitoring surveys. However, the fact that the spores are persistent in the environment, and thus accumulate, renders analysis for C. perfringens in sediments inappropriate as a basis for providing regulatory guidelines.

TISSUE

Sampling and analysis of shellfish tissue present fewer problems than sampling and analysis of water and sediment (see APHA 1985a). Shellfish sampling is very important because the consumption of shellfish as food, sometimes in the raw state, may present a serious public health hazard.

Shellfish offer several advantages for sampling: they concentrate bacteria, can be sampled relatively easily, and reflect pollution levels over relatively long periods in both sediment and water. In Puget Sound it is recommended that one or several shellfish species of recreational or commercial importance be sampled routinely at each major harvesting area. The use of a small number (preferably one) of species as standards will reduce the variation among stations and sampling periods that results from interspecific differences in the propensity to concentrate bacteria. Because the whole organism is eaten, the whole body should be prepared for analysis.

USES AND LIMITATIONS OF RECOMMENDED BACTERIAL INDICATORS

Recommended bacterial indicators for monitoring in Puget Sound are presented in Table 3 for each kind of sample matrix.

FECAL COLIFORM BACTERIA AND FECAL COLIFORM BACTERIA/E. COLI

The density of fecal coliform bacteria has commonly been used as an indicator of fecal pollution and as an indicator of the presence of pathogens. The widespread use of fecal coliform bacteria as an indicator has the advantage of providing a basis for comparisons with historical data. However, there are several distinct, and often substantial, limitations to using fecal coliform bacteria for these purposes. In addtion, the densities of coliform bacteria may not accurately reflect public health risks (Hanes and Fragala 1967). Recent research has indicated that, although many species of non-pathogenic enteric bacteria (e.g., coliform bacteria) are viable in aquatic systems, they are not totally recoverable using conventional techniques (Roszak et al. 1984; Xu et al. 1982). Normal culturing techniques may seriously underestimate the concentrations of these organisms in the environment. Because the degree of recoverability may also depend on variable environmental factors (e.g., nutrient concentrations), it may be difficult to develop general bacteriological standards that could apply to all areas.

Cabelli et al. (1983) concluded that fecal coliform bacteria were inferior to enterococci as indicators of the presence of pathogens in marine recreational waters with respect to evaluating public health risks. Survival times for coliform bacteria are substantially less than for many pathogens (Borrego et al. 1983), complicating efforts to correlate counts of fecal coliform bacteria with the densities of pathogens at any specific time. Another problem associated with the use of fecal coliform bacteria as indicators is the fact that they are not specific to mammalian fecal pollution. For example, the fecal coliform bacterium Klebsiella is common in pulp mill effluents.

For all of the above reasons, data on fecal coliform bacteria cannot in themselves be considered adequate for a thorough assessment of public health risks.Fecal coliform bacteria continue to be used as an indicator because other indicators also have deficiencies, and because measurements of fecal coliform bacteria provide a basis for comparisons with historical data. The lack of specificity of fecal coliform bacteria to mammalian fecal pollution prompted the development of a membrane filtration method for enumerating E. coli (Dufour et al. 1981). Unlike fecal coliform bacteria as a group, E. coli are specific to mammalian fecal pollution. The

TABLE 3. RECOMMENDED BACTERIAL INDICATORS FOR MONITORING IN PUGET SOUND

	MATRIX		
Objective	**Water[a]**	**Sediment**	**Tissue**
Recreational use evaluation (e.g., swimming)	Fecal coliform bacteria Enterococci	—	—
Pollution monitoring and/or water quality surveys	Fecal coliform bacteria E. coli C. perfringens Enterococci	Fecal coliform bacteria C. perfringens	Fecal coliform bacteria E. coli C. perfringens
Shellfish consumption evaluation	Fecal coliform bacteria[b]	—	Fecal coliform bacteria

a Fecal coliform bacteria are recommended here because current water quality standards are based on them. If these standards change to include only enterococci, it may still be useful to measure fecal coliform bacteria to maintain continuity with historical databases.

b U.S. EPA and National Oceanic and Atmospheric Administration (NOAA) currently are co-sponsoring research into the application of the enterococci and E. coli indicators for shellfish harvesting waters.

enumeration method incorporates a technique for distinguishing E. coli from other fecal coliform bacteria. The result is notated as fecal coliform bacteria/ E. coli. However, with respect specifically to recreational water quality criteria, U.S. EPA has not recommended the use of E. coli for marine and estuarine waters.

Laboratory analyses of fecal coliform bacteria are conducted using one of two most probable number (MPN) techniques (i.e., EC and A-1) or using a membrane filtration (MF) technique. The statistical reliability of the MF technique is greater than the MPN procedures (APHA 1985b). However, factors such as turbidity may reduce counts for the MF technique (Berger and Argaman 1983). Thus, the results generated by the MF and MPN techniques may not be directly comparable, and their inconsistent use by Puget Sound organizations limits comparisons of data gathered by different agencies.

ENTEROCOCCI

Enterococci are streptococcus bacteria indigenous to the intestines of warm-blooded animals. U.S. EPA recommends their use as indicators of fecal pollution in recreational waters because of the previously mentioned limitations of fecal coliform bacteria analysis, and because of the following characteristics:

- Because the concentration of enterococci has a greater correspondence to the incidence of gastroenteritis than do concentrations of E. coli or fecal coliform bacteria, it is a better indicator of public health risk in recreational marine waters (Cabelli et al. 1983).

- Enterococci may die off more slowly in sediments than fecal coliform bacteria (Van Donsel and Geldreich 1971), and therefore be better indicators of sediment contamination.

- Because they are tolerant to high salinity, enterococci are of particular value in analysis of marine waters (Coler and Litsky 1976).

- Taxonomic identification of streptococcus bacteria can be undertaken easily (e.g., API biochemical strips) and, unlike the situation with fecal coliform bacteria, can reveal the kinds of mammalian pollution (e.g., humans, livestock). This advantage arises from the fact that particular kinds of mammals harbor characteristic species of streptococcus bacteria (e.g., S. bovis in cattle).

- Techniques of gene fingerprinting (DNA analyses) have been undertaken using streptococcus bacteria and can more positively link bacteria in the environment to identical bacteria found in source effluents, thereby confirming the source(s) of contamination. Although not suitable for routine monitoring surveys, genetic analysis may be useful in certain research applications.

CLOSTRIDIUM PERFRINGENS

C. perfringens is consistently associated with fecal wastes and provides a usable, state-of-the-art marker for delineating the deposition and/or movement of sewage particulates that is more reliable than the traditional coliform bacteria indicators (Emerson and Cabelli 1982). *C. perfringens* is recommended for water, sediment, and tissue because it is present in wastewater at concentrations of 10^3-10^4 per 100 mL (Fujioka and Shizumura 1985), and because its resistance to chlorination and environmental factors closely resembles that of enteric viruses (Bisson and Cabelli 1980).

LABORATORY PROCEDURES FOR RECOMMENDED BACTERIAL INDICATORS

Recommended laboratory procedures for the bacterial indicators listed in Table 3 are summarized in Table 4.

TABLE 4. RECOMMENDED LABORATORY PROCEDURES FOR BACTERIAL INDICATORS

	Laboratory Procedures		
Test Organisms	Water	Sediment	Tissue
Fecal coliform bacteria	MPN tubes using A-I broth (APHA 1985b) (fecal coliform bacteria/100 mL)	MPN tubes using A-I broth (APHA 1985b) (fecal coliform bacteria/100 mL)	MPN tubes using EC broth (APHA 1985a) (fecal coliform bacteria/100 mL)
Fecal coliform bacteria/E. coli	mTEC (DuFour et al. 1981) (E. coli/100 mL)	—	—
Enterococci	mE (Levin et al. 1975) (enterococci/100 mL)	—	—
C. perfringens[a]	MPN tubes using iron milk (St. John et al. 1982) (C. perfringens/100 mL)	MPN tubes using iron milk (St. John et al. 1982) (C. perfringens/100 g)	MPN tubes using iron milk (St. John et al. 1982) (C. perfringens/100 mL)

a Two laboratory techniques are available for C. perfringens: mCP by membrane filtration for water (Bisson and Cabelli 1979) and sediment (Emerson and Cabelli 1982), and iron milk tubes using MPN techniques (St. John et al. 1982). The latter method is recommended (pending any comparative data) because the procedure is simpler and less costly.

QUALITY ASSURANCE/QUALITY CONTROL (QA/QC)

Laboratory and analytical QA/QC procedures are discussed in detail in U.S. EPA (1978) and APHA (1985b). Special problems exist in microbiological analyses because analytical standards, known additions, and reference samples generally are not available. However, a minimum QA/QC program should include:

- Ten percent of the total number of samples analyzed in duplicate
- Ten percent of the total number of samples split and analyzed by two or more laboratories
- Sterile distilled water transported to the field, transferred to a sample bottle, and processed routinely to ensure samples were not contaminated during collection and transport
- Repeated sampling at one site during varying conditions (e.g., tides, weather) to evaluate variability in the field.

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